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Somatic cell nuclear transfer as a tool for investigating ageing processes in mammals

Review Article

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Summary

The development of nuclear transfer technology has opened up a new frontier in the investigation of the processes which contribute to ageing in mammals. This review seeks to assess the individual hypotheses that have been proposed to account for the development of the ageing phenotype and to ask how they correlate with observations made on cloned mammals. In sheep derived by nuclear transfer there appears to be prematurely shortened telomeres, indicative of increased age. The animals, however, are physiologically normal, consistent with a redox model of ageing where mitochondrial damage is the key contributory factor. The application of nuclear transfer technology to the study of ageing phenomena and its use in experimentally redressing aspects of the ageing phenotype is discussed.

I. Introduction

Ageing can be defined as an increase in molecular chaos over time. This is generally manifest as a change in phenotype and an associated exponential increase in the likelihood of mortality. The latter part of that definition was first described by Gompertz (1825), but it has only been in more recent times that the molecular and cellular events giving rise to the age related phenotypic changes have begun to be unraveled. The seminal work of Hayflick and Moorehead (1961) in describing replicative senescence for human fibroblasts *in vitro*, gave a new impetus to studies in gerontology and has paved the way for a description of the molecular basis of ageing.

Contemporary investigations in the nematode *Caenorhabditis elegans* and in lower eukaryotes, principally *Saccharomyces cerevisiae* (reviewed by Guarente, 1997), have given direct insight into which genes and molecular processes underlie the basis of ageing at the cellular level and provide a basic paradigm for ageing in higher organisms. The most celebrated model of ageing is still, however, based upon *in vitro* senescence data and centres around telomere shortening as a molecular clock. Other molecular models have been proposed, based on oxidative damage and fragmentation of mitochondrial DNA (reviewed by Ozawa 1997; Osiewacz, 1997), or upon age dependent demethylation of DNA. These are not necessarily mutually exclusive, but all require critical testing *in vivo*.

Nuclear transplantation provides a powerful tool for examining the relationship between age related changes at the molecular level and ageing in the whole animal. The advent of Dolly, derived by nuclear transplantation of an adult nucleus from a mammary cell of a six year old animal (Wilmut et al, 1997), provides for the first time, the ability to directly test these models in an *in vivo* context. The facility to recreate a higher organism while circumventing the germline and potentially, the resetting of any molecular clocks, provides a new approach to the investigation of the relationship between factors determining physiological age and cellular senescence.

II. Models of ageing

A. Telomere based clocks

The telomere hypothesis of cellular ageing (Olovnikov, 1973; Cooke and Smith, 1986; Harley, 1991; Bodnar *et al*. 1998) espouses that the loss of telomeric DNA through incomplete replication of chromosome ends and lack of telomerase to repair damage, provides a mitotic clock that

eventually signals cell death. Once a critical loss of telomeric sequences has occurred from the chromosome ends, replicative capacity is compromised and the cell dies. Telomere shortening has been causally implicated in human cellular senescence (Harley et al 1990; Allsopp et al, 1992), disease (Oexle and Zwirner, 1997) and by general implication, the physiological ageing process in higher animals.

Telomeres are specialised structures found at the end of eukaryotic chromosomes, consisting of simple repetitive DNA; in mammals telomeres comprise $(TTAGGG)_n$ (Moyzis et al, 1988). They have at least three roles in the maintenance of chromosome structure and integrity, (i) a capping function that is to protect DNA ends from fusion, recombination and degradation, (ii) attachment of the chromosome to the nuclear envelope and (iii) facilitation of the complete replication of chromosome ends (Olovnikov 1973). In man, the latter is achieved via the mediacy of a unique ribonucleoprotein complex, termed telomerase, which has the capacity to synthesise telomeric DNA *de novo* onto the 3' end of the parental G rich strand, using the telomerase RNA component as template. This then allows DNA polymerase to conventionally complete the synthesis of the daughter strand. Telomerase principally functions in the human germ line, stem cells and haemopoeitic cells, but not in somatic tissues where telomere damage accumulates over time. Consequently, telomeres in man shorten during replicative ageing (Cooke and Smith, 1986; de Lange, 1992; Frenck et al, 1998).

The telomere capping function appears to be mediated by a combination of a unique tertiary structure and specific telomere binding proteins. Conventionally, this was thought to be achieved either by a specific DNA binding protein attaching to the linear chromosome end (Gottschling and Zakian, 1986) or by a distinct structure composed of a quartet of G residues at the single stranded terminus of the telomere (Williamson et al, 1989). As yet, there has been no direct *in vivo* evidence for either of these features in higher eukaryotes.

Electron microscopy has shown that the mammalian telomere takes the form of a loop, termed the t-loop, created by the telomere DNA folding back on itself to form a lariat whose leading end is the telomeric 3' G strand overhang. This is envisaged as invading adjacent duplex telomeric repeats, thus creating a displacement loop (D loop). Duplex DNA binding proteins are proposed to bind along the telomeric repeats of the t-loop, while a specialised DNA binding protein stabilises the D-loop lariat junction (Griffith et al, 1999). This is illustrated in **Figure 1**.

Reconciliation of a telomere based model of ageing and cellular senescence with a telomere structure based on a t-loop, poses some intriguing questions.

Does, for example, encasement within the duplex serve to distinguish the terminal sequence from damage accrued DNA breaks? Physical isolation of the telomere from normal DNA damage responses would have a number of implications for models seeking to explain the loss and gain of telomeric DNA.

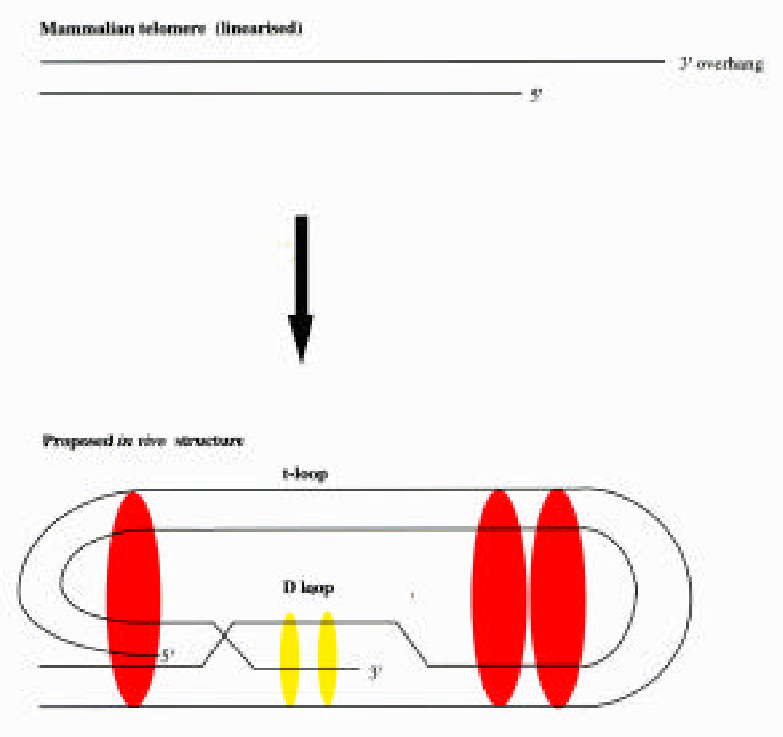

Figure 1. Hypothesised structure for mammalian telomeres. The telomere is proposed to loop back upon itself with the 3' overhang invading the adjacent duplex creating a displacement loop (D-loop) which is stabilised by specific telomere binding proteins (yellow ovoids). Other duplex DNA binding protein complexes (red ovoids) are proposed to engage along the t-loop stabilising the whole structure.

In the first instance, the presence of the 3' G strand extension of the telomere within the telomeric duplex, can be construed as being representative of an intermediate event during the process of recombination. This could theoretically potentiate DNA loss via branch migration and subsequent degradation of single stranded DNA segments. Gain of telomeric DNA is also possible if the 3' invading strand initiates the priming of de novo DNA synthesis subsequent to strand invasion. These mechanisms are not without precedent . Two alternative mechanisms to initiate DNA replication, one dependent on Escherichia coli RNA polymerase the other dependent on general recombination, have been reported for bacteriophage T4 (Luder and Mosig, 1982). Such mechanisms may contribute to telomere dynamism, particularly in the context of telomerase negative cells, where alternative telomere lengthening (ATL) mechanisms have been reported (Kipling. and Cooke 1990; Starling *et al*, 1990; Prowse and Greider, 1995).

A loop structure also has consequences for telomerase function. How, for example, does telomerase gain access to the terminal sequence? It is not intuitive that having a terminal sequence buried within a D loop and stabilised by bound proteins is a readily accessible substrate. Does this singular structure act as a beacon for the telomerase holoenzyme? The implication is that for telomerase to access the terminus, there may be inherent dynamism in the loop during replication.

B. The nucleolus and ageing

Analysis of senescence in *Saccharomyces cerevisiae* has led to the development of a strikingly simple model to explain senescence in yeast, which may be applicable to ageing in higher organisms. Mutants for the *SGS 1* gene, which codes for a yeast Rec Q-like helicase, senesce prematurely and show a characteristic accumulation of extrachromosomal rDNA circles in mother cells following successive asymmetric cell divisions, which leads to cell death (Sinclair and Guarente, 1997).

The accumulation of extrachromosomal rDNA circles is accompanied by nucleolar fragmentation (Sinclair et al 1997) and a disruption of silencing complexes at telomeres and HM silent mating type loci. These silencing complexes are composed of Sir proteins and appear to promote longevity. Deletion of component members, such as Sir 3 or Sir 4 results in shortened lifespan. A consequence of their disruption is the relocation of Sir 3 and Sir 4 proteins into the nucleolus (Kennedy et al 1997). Whether this is a direct response to the accumulation of the rDNA circles or events associated with their formation, is not fully determined.

The telomeric location of Sir protein complexes and their involvement in regulating telomere length and telomeric silencing, coupled with a role in ageing, is intriguing, especially in view of the correlation between telomere shortening and ageing in mammals. In *S. cerevisiae*, however, telomeres do not shorten with age, and an inverse correlation between telomere length and lifespan has been observed (Austriaco and Guarente1997). Conversely, telomere length in yeast correlates positively with telomere silencing. In the absence of telomere erosion with age, longer telomeres could be envisaged as better competitors for silencing complexes, with resultant exacerbation of rDNA circle generation. These observations are not irreconcilable with observations in mammals where telomere shortening occurs with increasing age. In this instance the release of Sir complex analogues for recruitment to nucleolar or other sites would result as a consequence of telomere erosion. Thus when viewed from the perspective of the whole organism, telomere shortening, rather than acting as a molecular clock in mammals, may be a homeostatic mechanism to prolong lifespan. As yet, there are no reports of extrachromosomal rDNA circles for ageing mammalian cells. It will be interesting to see if mammalian species with longer lifespans do indeed all have relatively longer telomeres.

Significantly, SGS1 is the homologue of *WRN* in man, the gene responsible for Werners syndrome, a premature ageing condition, hence the possibility that a similar derangement of rDNA sequences is contributory to human ageing. Interestingly, the WRN protein has a central domain which is homologous to members of the RecQ family of DNA helicases and has been shown to catalyse DNA unwinding (Gray et al 1997), which is in keeping with the role of *SGS 1* in yeast.

C. Oxidative damage and ageing

Ageing as a consequence of cumulative molecular insults has formed the basis of a number of models over the past 40 years. DNA based models of ageing (Alexander 1967; Ozawa, 1995.1997) operate on the premise that oxidative damage to DNA should increase with age and result in decreased functional capacity, inclusive of the ability to repair oxidative damage. By extension, models which propose that the principal agents of DNA damage are free radicals generated as a by-product of oxidative metabolism would predict that mammals with lower metabolic rates should have an increased lifespan and a decreased rate of accumulation of somatic damage compared to those with higher metabolic rates. Experimental evidence in support of such a hypothesis is widespread, though circumstantial. An inverse correlation between lifespan and metabolic rate has been observed in mammals (Cathcart et al 1984). For exaple, rats who have a higher metabolic rate than man, have a shorter life span and a higher rate of accumulation of free radical engendered DNA damage. These differences are more pronounced in the mouse, whose lifespan is even shorter and metabolic rate higher than

that of the rat. Analysis in monkeys again correlates well with this hypothesis (Adelman et al 1988).

Rodents do show a comparatively higher age related increase in DNA oxidative damage products, such as 8-hydroxy-2'-deoxyguanine (8-OHdG) (Fraga et al 1990: Sohal et al 1994). Significantly, the levels of such DNA damage products can be reduced by calorific restriction, which has been shown to increase longevity in rats and mice (Sohal et al 1994). Dietary restriction would be expected to have an influence on a wide range of genes (Mote et al 1991), including those involved in oxidative metabolism (e.g. superoxide dismutase, catalase) through the generation of cellular stress responses. Indeed, cells from calorifically restricted animals maintain replicative potential longer *in vitro* than those from *ad librum* fed controls, which is consistent with a loss of replicative potential *in vivo* being associated with cumulative oxidative insults (Hass et al, 1993).

Studies on cells derived from progeric Werners syndrome patients are also consistent with a model, in which the products of oxidative damage accumulate through lack of sufficient DNA repair. Cells from these patients display hyper-recombination, increased mutation frequency and a propensity for large deletions (Fukuchi et al 1989; Cheng et al 1990). WRN patients also present with a high incidence of rare malignancies (Goto et al 1996), consistent with a defect in DNA repair processes which probably relates to the helicase function of the WRN protein (Gray et al 1997). This is supported by observations on another progeric condition, Cockayne's syndrome, which similarly displays characteristic defects in DNA repair (van Gool et al 1997).

Most DNA repair syndromes, however, do not show features typical of progeria and, in contrast to the above observations, it has been reported that *in vitro* DNA repair capacity is not affected by age (Kunisada et al 1990). These authors studied the repair capacity of human fetal lung fibroblasts and primary embryo fibroblast cultures from rat lung and skin, for their capacity to repair a reporter plasmid which had been UV irradiated prior to transfection. Neither age-related, nor change as a function of passage number was found in the repair of UV damage in these cells.

These data remain to be reconciled with previous observations, as they appear counter-intuitive. The data may reflect elevated stress responses as a consequence of cell culture or, more significantly, reflect DNA repair capacity in relation to the stage of senescence of immortalised cells *in vitro* or senescing primary cell cultures. It may not be sufficient to extrapolate these observations to *in vivo* age related changes in DNA repair capacity.

A stronger correlate for models of ageing based on oxidative damage can be found in the examination of mitochondrial DNA (mtDNA). A decrease in mitochondrial respiratory activity and an increase in mitochondrial mutations and fragmentation has been positively associated with increasing age (Linnane et al, 1989,1990; Hayakawa et al 1992). Oxidative damage to mtDNA may have more pronounced effects, as mitochondria in post mitotic cells maintain the capacity to replicate (Menzies and Gold 1971), hence, the potential exists to generate and accumulate deleterious mutations which become fixed in the cell population and lead to respiratory deficiency and degeneration. These ideas have been incorporated into "the redox mechanism of ageing" (Ozawa 1995,1997), which hypothesises a molecular basis for the age related decline in cellular activity, tissue and organ degeneration and age associated deterioration in cognitive performance. Mitochondrial mutations are proposed to arise afresh each generation and accrue with age. These mutations are proposed to correlate directly with oxidative damage and cell death. The level of accumulated mutations is considered to directly equate with age related decline in cellular function.

While many aspects of this hypothesis seem intuitive, evidence in support of it still remains largely circumstantial. The pronounced age related accumulation of 8-OHdG in mtDNA relative to nuclear DNA (Hayakawa et al 1992) and a correlation with a decline in the mitochondrial electron transfer chain (Hayakawa et al 1993; Takawasa et al 1993) support the hypothesis.The results of elevated stress resulting from mitochondrial mutations have also been observed clinically in patients with mitochondrial myopathy (Ozawa et al 1995) and in murine models of mitochondrial disease (Esposito et al 1999).

Direct confirmation of this hypothesis at an organismal level and its integration with other models of ageing, is a facinating prospect. It is not immediately obvious, for example, how redox theories of ageing can be incorporated into a genetic model of ageing, such as that for the klotho mouse (Kuro-o et al 1997). The latter is especially intriguing as the mouse presents with a progeric syndrome with many features in common with human ageing including infertility, arteriosclerosis, osteoporosis, emphysema and skin atrophy. Surprisingly, these all result from the function of a single mutant gene with similarity to β-glucosidase. Whether the klotho mouse actually represents a true ageing syndrome or is simply a good model for diseases of ageing is undetermined. The investigation of such a genetic model, in the context of current hypotheses of ageing, should prove worthwile given that only a single gene is responsible for the klotho phenotype.

D. DNA methylation and ageing

The rate of loss of DNA 5-methyldeoxycytidine residues appears to be inversely related to lifespan (Wilson and Jones 1983; Wilson et al, 1987). Losses in genomic 5-methyldeoxycytidine content have been observed to correlate with donor age in cultured normal human bronchial epithelial cells and *in vivo* derived murine genomic DNA (Wilson et al, 1987). Conversely, the level of DNA 5-methyldeoxycytidine appears relatively stable in immortalised cells. Significant losses of DNA 5-methyldeoxycytidine residues in old age could alter cellular gene expression and contribute to the physiological decline of the animal. Treatment of cells with agents that induce random hypo-methylation induce premature senescence (Gray et al 1991).

This correlation between accelerated DNA demethylation and accelerated ageing, while suggesting that these two phenomena are related, does not indicate direct causation. DNA demethylation during ageing may not be random, and could co-operate with other independent ageing processes to produce a finite lifespan and age associated phenotype. In both instances, accelerated DNA demethylation could advance ageing, though *in vivo* this may not be a reflection of the overall level of genomic 5 methyldeoxycytidine, but rather the perturbation of function of specific gene(s). Furthermore, methylation is thought to stabilise heterochromatin and loss of methylation with age *in vivo*, or *in vitro* , may correlate with the loss of telomeric heterochromatin. As such, age related loss of methylation is not incongruous with other models of ageing, in particular those involving erosion or destabilisation of telomeres.

III. *In vivo* analysis of ageing.

Analysis of *in vivo* ageing has never been straight-forward. Critical testing of the various hypotheses to account for ageing in eukaryotes has generally relied on inter-generational comparisons or between mutant and wild type animals. Many investigations simply extrapolate from *in vitro* data. Such experiments have produced a wealth of information on the molecular processes involved in ageing, but they cannot be extricated from the influence of endogenous molecular clocks (e.g. telomere length), variation in genetic background and artefacts arising from *in vitro* senescence phenomena. Consequently, the ability to distinguish between the relative contributions of genetic and structural damage, a reasonable prerequisite for the formulation of an accurate model of *in vivo* ageing, is not readily addressed in previous analyses. Accordingly, a comprehensive and integrated determination of the contributions of the individual molecular processes to the ageing phenotype has not been achieved.

The development of nuclear transfer (NT) using cultured somatic cells (Campbell et al, 1996; Wilmut et al, 1997; Schnieke et al., 1997; Wells et al , 1997; Ashworth et al., 1998; Signer et al., 1998; Cibelli *et al.*, 1998; Wakayama et al 1998) offers a new analytical approach. It advances the possibility of viewing age related changes, both at the single cell level and at the level of the whole organism, against a uniform genetic background with circumvention of any molecular clock(s) reset in the germline. Importantly, it allows dissection of the relationship between ageing processes at both these levels.

Aspects of the nuclear transfer procedure impinge directly upon the central tenets of current theories of ageing which can now be subject to integral analyses. Critical to such analyses is the ability to compare clones at different chronological ages, either *in vivo*, or *in vitro*, in order to assay directly age related phenomena. Significantly, this is independent of the age of the progenitor tissue. The capacity to serially derive animals (i.e.: clones of clones) by NT (Wakayama and Yanagimachi, 1999) and to genetically manipulate the cell prior to nuclear transplantation (Schnieke et al 1997), increases the power of the possible investigations. This offers the capability of restoring telomerase activity to previously telomerase negative cells, knocking out or mutating genes implicated in the ageing process, such as klotho, free radical scavengers or nuclear encoded mitochondrial genes, to name but a few.

Importantly, nuclear transfer results in the separation of the nucleus from the mitochondria of the progenitor cell during the transplantation procedure. The relative contributions of genomic and mitochondrial damage and how these are manifest in the ageing organism can now be addressed.

The obvious sequitur from the use of nuclear transfer as a tool to investigate ageing processes will be to ask if any of these processes are reversible. For example, can telomere erosion be repaired? Can one mitigate the effects of nuclear oxidative damage? Can mitochondrial function be restored?

A. Mammalian clones

To date four mammalian species have been used to successfully derive clones by nuclear transplantation; these comprise sheep (Campbell et al, 1995; Wilmut et al, 1997), cattle (Wells et al 1997; Cibelli et al 1998), mice (Wakayama et al 1998) and goats (Baguisi et al 1999). The methodological considerations and general applications of cloning have been reviewed extensively elsewhere (Campbell 1999, Colman 1999). An outline of the nuclear transfer procedure is shown in **Figure 2**.

The creation of Dolly (Wilmut et al, 1997) was of particular significance to studies of ageing in that the progenitor nucleus was not only derived from a somatic cell type, but from a six year old adult. Whilst these authors were unable to confirm whether the cell had a fully differentiated phenotype, subsequent studies using adult cells from cows did

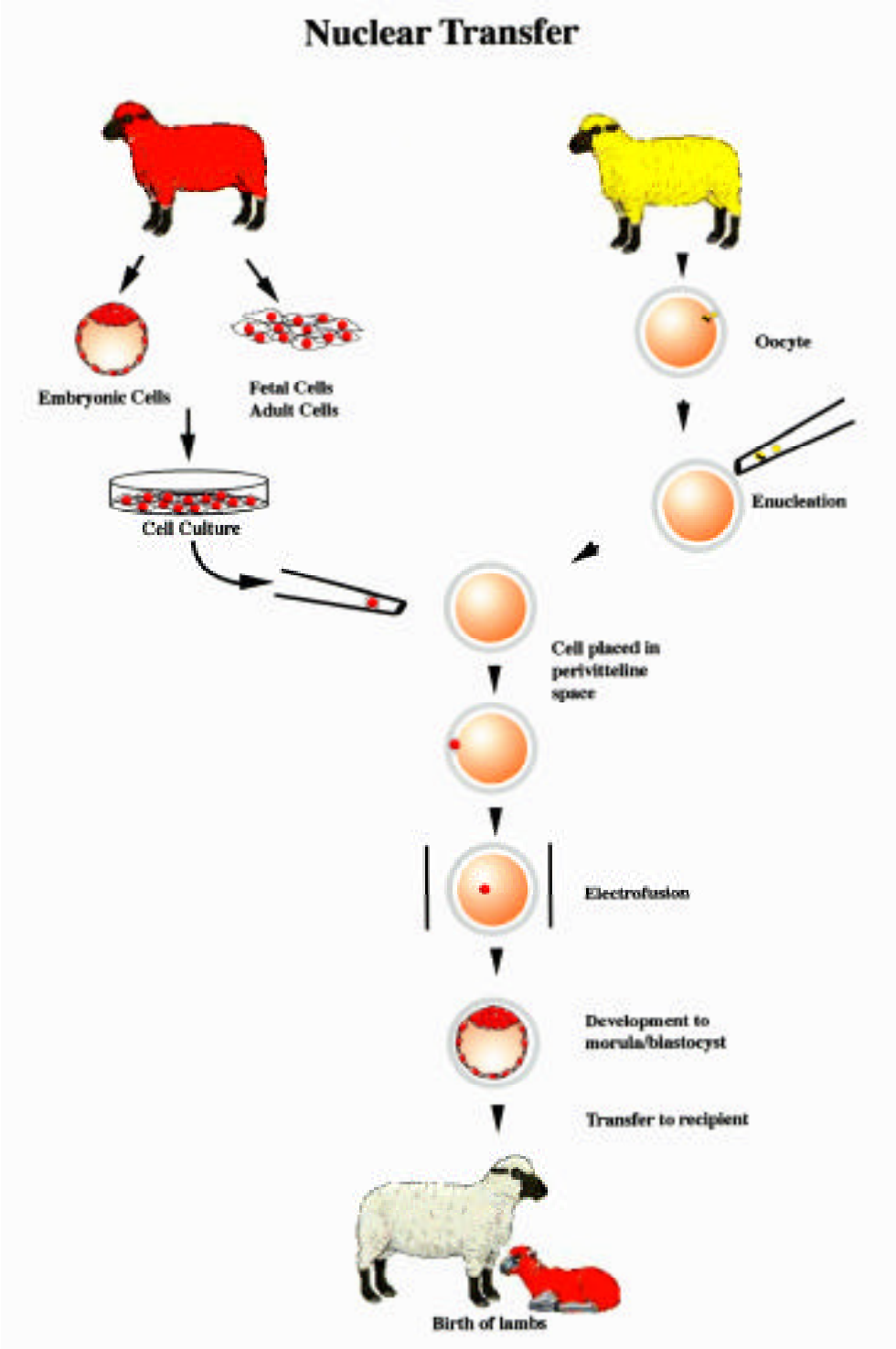

Figure 2. Schematic outline of the nuclear transfer procedure. Oocytes derived from Scottish Blackface (symbolised as a yellow sheep) are enucleated. The donor cell derived from a different sheep breed (symbolised as a red sheep) is placed under the zona pelucida into the perivitelline space. The cell nucleus is introduced into the cytoplast by electrofusion, which also activates the oocyte. The reconstructed embryo is then either cultured *in vitro* up to blastocyst stage or is transferred into a pseudopregnant intermediate recipient ewe. At day 7 embryos are assessed for development. Late morulae and blastocysts are transferred into final recipients. Pregnancies resulting from nuclear transfer are determined by ultrasound scan at about 60 days after oestrus and development is subsequently monitored at regular intervals.

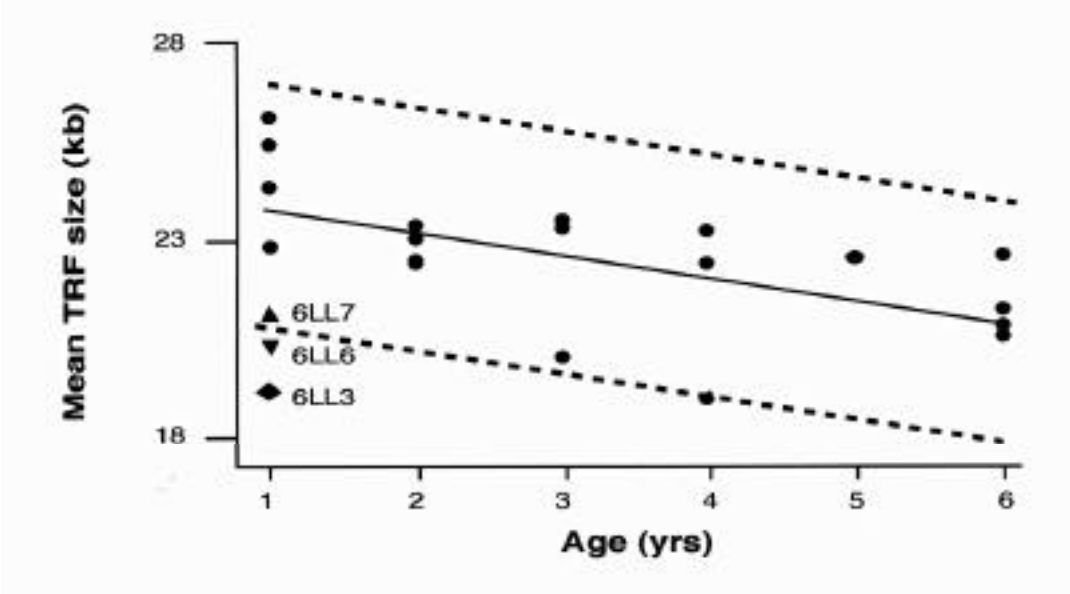

Figure 3. Regression analysis of mean TRF lengths in NT sheep and controls. Graph showing the telomere length decline with age for control sheep (solid circles) and NT animals together with the fitted line (solid) and 95% prediction interval for an additional observation at any given age (dashed line).

use differentiated cells (Wells et al 1999). Adult somatic cells have also been used to derive clones in mice (Wakayama et al 1998).

B. Observations on ageing in ovine clones.

Initial investigations into ageing in cloned animals has revolved around an examination of telomeres in sheep (Shiels et al 1999a, 1999b). These experiments asked if the generation of animals without germline involvement results in the resetting of telomere lengths and hence any molecular clock which measured time by such lengths.

Three cloned animals were examined, whose derivation spanned distinct developmental stages and cell types. These comprised animal 6LL3, ("Dolly"), derived by transfer of a nucleus from ovine mammary epithelial (OME) cells from a 6 year old sheep, 6LL6 from sheep embryonic cells (SEC 1) obtained from day 9 embryos, and 6LL7 which was derived from fibroblasts from a day 25 fetus. All three animals showed apparent telomere diminution as determined by measurement of mean terminal restriction fragment (TRF) lengths. The TRF diminution observed in 6LL3 was the greatest of the three animals and was consistent with the age of her progenitor ovine mammary tissue (6 years old) and significantly the time OME cells derived from that tissue, spent in culture prior to nuclear transfer.

The influence of time spent by donor cells in culture is substantial. Telomere shortening due to enhanced damage attributed to reactive oxygen species (ROS)*in vitro*, has previously been reported (Von Zglinicki *et al* , 1995; Zijlmans*et al*., 1997). The full effect of such oxidative damage, however, is only manifest subsequently, in any clone derived from such cells. The contribution of *in vitro* culture to telomere erosion in sheep derived by nuclear transfer, superimposed on the age of progenitor tissue, could be gauged from the TRF diminution of OME cells that had undergone up to 27 population doublings (9 passages)in culture. When compared to the mammary gland from which these cells derived and to Dolly, derived in turn from these cells after 3 population doublings, a mean TRF decrease was observed at an average 0.157 kb per population doubling. The immediate implication of these observations is that the extent of TRF shortening can be mitigated, principally by minimising time in culture and the age of donor cells. This is particularly relevant with respect to animal 6LL7, where the use of fetal tissue and minimal culturing yielded an animal where the mean TRF size is not significantly shorter than age matched controls, unlike 6LL3 and 6LL6 where culturing was more prolonged (**Figure 3**).

The most likely explanation for the shorter mean TRF lengths of all three nuclear transfer sheep is that the TRF size observed reflects that of the transferred nucleus. Whether this telomere erosion is reflected in the overall ageing process of these animals is uncertain. It is not known whether the actual physiological age of animals derived by nuclear transfer is accurately reflected by TRF measurement. No physiological progeria has yet been reported in any animal derived by nuclear transfer. Veterinary examination of the cloned animals has confirmed that they are healthy and typical for sheep of their age and breed, despite having a shorter mean TRF length.

Furthermore, 6LL3 has undergone two normal pregnancies and successfully delivered healthy lambs.

The telomere hypothesis of ageing (Olovnikov, 1973; Cooke and Smith, 1986; Harley, 1991; Bodnar *et al* , 1998), however, would predict that animal 6LL3 would reach a critical telomere length sooner than age matched controls. However, ovine TRFs show a large size distribution, from 5-50Kb (Shiels et al 1999a,b), thus it remains to be seen whether a critical length will be reached during the animal's lifetime. Experimental inactivation of murine telomerase only produced a phenotype after five generations (Blasco et al 1997; Lee et al 1998) and similar observations have been made in telomerase deficient yeast cells (Lundblad and Blackburn, 1993).

It is noteworthy, in respect of the inverse correlation between telomere length and lifespan, that the TRF spread in sheep appears to fall between those of mouse and man in accord with the hypothesis that longer telomeres mean a shorter lifespan.

Clarification of whether telomere erosion is causative for, or an effect of the ageing process is not immediately apparent. The observations on cloned sheep are, however, congruent with the redox theory of ageing (Ozawa 1995,1997) which would predict that the vigour and fecundity of such animals would be physiologically identical to age matched controls.

IV. Cause and effect: testing the models of ageing.

Mitigation of the observed effects of both ageing and *in vitro* senescence do seem feasible. It is this capacity which will allow dissection of the component parts of the ageing process and illustrates the potential of nuclear transfer as a tool to achieve this. Strategies for remedial action can be detailed as follows.

A.Telomere erosion

Introduction of telomerase to normal human cells in culture has been reported to significantly increase their lifespan (Bodnar *et al*, 1998).

Ectopic expression of the telomerase catalytic subunit (hTERT) and subsequent activation of telomerase in postsenescent cells has been demonstrated to allow the cells to proliferate beyond crisis (Counter et al 1999). Furthermore, alteration of the carboxyl terminus of hTERT appears not to affect telomerase enzymatic activity, though it prevents telomere maintenance and consequent cell proliferation. Cells expressing hTERT ectopically appear phenotypically normal and exhibit no manifestations of malignant transformation (Jiang et al 1999; Morales et al 1999).

While these observations indicate a strong correlation between telomere erosion and the timing of cellular senescence, how this will be integrated within a complete description of the chronological ageing phenotype has yet to be determined. Any extrapolation beyond consideration of telomere length as one feature of a mitotic clock would seem to be premature.

While telomere erosion can be directly addressed experimentally, it has still to be fully established how such hTERT expressing cells will fare *in vivo*. A key consideration in this context , will be the effect of any genetic damage acquired *in vitro* (and for that matter *in vivo*) which is not accessible to telomerase repair. Another consideration is whether oxidative damage is primarily manifest as telomere erosion, due to telomere sequence content or cellular localisation.

A donor nucleus source which is naturally telomerase positive, such as a lymphocyte, might be a better choice to mitigate the effects of telomere erosion without recourse to genetic manipulation. Such a cell type, however, might be considered unsuitable as a donor source, as telomerase positivity is often a characteristic of malignancy. Telomerase positivity coupled with the increased risk of *in vitro* accrued oxidative damage, means that the chances of neoplastic transformation are increased. Quantification of this risk, however, is not straightforward and requires species specific model systems to address the issue (for a fuller discussion see Colman 1999). Suitable model systems have not yet been established.

Parenthetically, it is not known whether outbreeding a cloned animal or inter breeding clones will restore telomere lengths. In the former instance, the presence of the chromosome complement from the naturally derived parent would provide a haploid complement of full length telomeres, while in the latter germline resetting of telomere lengths would be required. It is unknown if germline resetting of telomeres occurs in interbred clones. This is presently being investigated.

Another aspect of telomere erosion in mammals that needs clarification is the relationship between this phenomenon and that of extrachromosomal rDNA circle generation as described in yeast (Sinclair et al, 1997). A comparison of Dolly with a relevant control panel of sheep should prove informative in this context, as the nucleolar model would predict that she should at least reflect the age of her progenitor tissue. This is supported by analysis of terminal restriction fragment lengths (Shiels et al, 1999a,b).

A final consideration with regard to telomeres is whether there is a causal relationship between age associated demethylation and telomere attrition.

If so, ameliorating telomere shortening may mitigate loss of methylation and equally the generation of extrachro-

mosomal rDNA circles. It remains to be determined if this hypothesis is valid.

B. Oxidative damage

While shortening time spent in culture offers an immediate route to decreasing any telomere erosion due to reactive oxygen species, this should also reduce genome wide oxidative damage. Calorific restriction offers a second means of mitigating oxidation effects, with a proven efficacy *in vitro* and *in vivo* (Hass et al, 1993; Sohal et al, 1994).

A number of issues pertaining to the source, extent and contribution of such oxidative damage can now be brought into focus. Mitochondrial damage contributing to the overall ageing process, as postulated within "the redox mechanism of ageing" (Ozawa, 1995, 1997) is both amenable to direct investigation and redress. Mitochondria in cloned animals almost entirely derive from the recipient oocyte cytoplasm (Evans et al 1999), hence problems arising as a consequence of the higher mutation rate in mitochondria and their accumulation, particularly in post-mitotic cells, are circumvented. If the tenet of this hypothesis is valid then one would expect animals derived by nuclear transplantation from adult cell sources not to show the physiological characteristics of the age of their progenitor, but to reflect that of age control animals. Only genomic damage acquired by the donor nucleus should be transferred to clones and manifested accordingly.

The physiological characteristics of Dolly and other sheep clones (Wilmut et al 1997; Shiels et al 1999a,b) and the failure to detect premature ageing in cloned mice (Wakayama et al 1999) are in keeping with such a hypothesis, whereby damage to mitochondria is a key event in the physiological degeneration associated with ageing.

Genomic damage inherited by the clones is not easily redressed. As the accumulation of deleterious somatic mutations correlates positively with increasing age, there is a greater likelihood of acquiring damage to developmentally important genes through the use of older donor cell sources. This obviates the practical choice of using nuclei from younger cell sources as donors for nuclear tranplantation.

Assessment of the relative consequences of mitochondrial and genomic oxidative damage and nuclear-mitochondrial communication within the ageing process, can also be addressed by manipulation of nuclear encoded mitochondrial genes and to some extent, by variation of the recipient cytoplasm. This should allow determination of to what extent accumulation of mutations in the nucleus contributes to age related mitochondrial dysfunction.

V. Conclusions.

Molecular gerontology is, if you excuse the pun, rapidly coming of age. Recent advances in nuclear transplantation technology (for a review see Colman 1999) have provided a direct route to the investigation of the individual processes that give rise to the ageing phenotype and for the first time allowed their direct testing *in vivo*. The manipulation of such processes, using the full panoply of molecular techniques and the powerful genetics available for studies in mice, should allow for a fuller understanding of ageing from the level of the single cell to the whole organism. The potential to mitigate the effects of these processes exists in the laboratory, though the practical application of this technology is not so easily achieved. One immediate application of this technology is the development of Cellular therapies for countering the effects of diseased or degenerating tissues. This would comprise the molecular manipulation of cells *in vitro* , repairing for example, telomere erosion, replacing deleterious alleles of age important genes, upregulating oxygen free radical scavrengers, or replacement of damaged mitochondria. Such cells could, for example, be used to supplement or replace cells in failing organs. Consequently, the functional replacement of degenerating tissues may no longer fall within the realms of science fiction.

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